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Occurrence and characteristics of residual follicles formed after transvaginal ultrasound-guided follicle aspiration in cattle

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ABSTRACT

Ultrasound-guided transvaginal follicle aspiration is used to recover cumulus-oocyte complexes (for IVF) and to synchronize follicular wave emergence (ablation of dominant follicle). Although aspirated follicles are generally supposed to undergo immediate atresia, there are indications that they may remain active. The objective was to evaluate the occurrence and characteristics of residual follicles (RF) after transvaginal follicle aspiration in cattle. Ovarian follicular wave emergence was synchronized in Holstein cows (N = 13) in the presence (groups 1 and 3) or absence (groups 2 and 4) of norgestomet implants. The largest follicle was aspirated at a diameter of 8 mm (groups 1 and 2) or 12 mm (groups 3 and 4). Ovarian follicles were visualized (transrectal ultrasonography) every 12 h after wave emergence. Follicular fluid samples were collected from the largest follicle and from the ensuing RF and concentrations of estradiol and progesterone were determined. After aspiration, 73.2% (52/71) of the follicles refilled with fluid, and a new antrum was detected 12 to 24 h later. Norgestomet did not affect ($P > 0.05$) RF occurrence or diameter, but in RF from group 4, concentrations of estradiol decreased (-530.7 ± 133.9 ng/mL; $P < 0.01$) whereas progesterone increased ($+429.6 \pm 171.7$ ng/mL; $P < 0.05$) relative to preaspiration. In RF, there were three steroidogenesis patterns: (1) high estradiol concentration and high estradiol:progesterone ratio (estradiol-active RF); (2) low estradiol, but high progesterone concentrations (luteinized RF); and (3) low estradiol and low progesterone concentrations (inactive RF). Estradiol-active RF were more likely ($P < 0.05$) from follicles with high estradiol concentrations (regardless of diameter). In conclusion, fluid-filled structures (RF) with variable steroid production patterns are frequently formed after ultrasound-guided follicle aspiration. The occurrence and features of these RF depended on the diameter and status of these follicles before aspiration.

1. Introduction

Ultrasound-guided transvaginal follicle aspiration, also known as ovum pick-up (OPU), has become the technique of choice for recovering cumulus oocyte complexes from live donors for IVF, both in cattle [1] and humans [2]. In essence, OPU is an adapted biopsy procedure in which ovarian follicles are visualized with real-time ultrasonography, and their content is aspirated using a needle connected to a vacuum system. The ultrasound-guided needle is a reliable and practical way to access follicles within the ovary; it has been used to evaluate the intrafollicular environment [3], to biopsy preantral follicles [4], and to perform intrafollicular injections [5].

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Follicular dominance is a key mechanism in ovarian follicular dynamics for establishment of the species-specific ovulation rate (reviewed by Ginther et al. [6]). In cattle, the appearance of a dominant follicle causes atresia of the subordinate follicles within the same follicular wave (reviewed by Ginther et al. [7]); negative effects of follicular dominance on superovulation are well documented [8–10]. Ultrasound-guided follicle aspiration became, consequently, an alternative to selectively remove large antral follicles and to control follicular dynamics [11], and was therefore used by various research groups to ablate the dominant follicle and thereby synchronize follicular wave emergence [12–14] or to improve superovulatory response [9,15–19]. Follicular dominance has also been demonstrated to negatively affect oocyte developmental potential [20–22]; therefore, short OPU intervals were proposed to avoid establishment of dominant follicles and improve IVF outcome [23–25].

Follicular aspiration causes follicle collapse and lumen disappearance, based on the ultrasound image [9]. The remaining follicular wall cells are generally supposed to undergo atresia after aspiration, but in most studies, the fate of the aspirated follicles was not recorded thereafter, and this expected immediate atresia remained a presumption. Ginther et al. [26] were apparently the first to describe refilling of follicle contents after aspiration, and hypothesized that active components of the follicle might not have been destroyed or removed. Subsequently, our group described this phenomenon as fluid-filled structures, larger than 6 mm in diameter, and with irregular fibrin clumps visible ultransonographically 12 to 24 h after initial collapse of aspirated follicles [27]. We also highlighted the possibility of the maintenance of steroidogenic activity by these residual follicles, after measuring estradiol and progesterone concentrations in fluid samples collected from these structures [27]. Interestingly, not much attention has been given to these refilled follicles. Such structures were reported only in a few other studies, and usually as a secondary observation [28]. Hayashi et al. [29] monitored dominant follicles aspirated at various intervals before or after GnRH treatment, but the focus was on CL development. To our knowledge, no specific study has been reported that addressed steroidogenic activity or the biological relevance of residual follicles formed after aspiration.

The aim of the present study was to evaluate the occurrence and steroidogenic activity of residual follicles formed after ultrasound-guided aspiration of the largest follicle in various diameters from cows, with or without exogenous norgestomet.

2. Materials and methods

2.1. Animals and location

This study was performed at Santa Monica Experimental Station, located in Valença, RJ, Brazil. Thirteen pluriparous, nonlactating, Holstein cows with regular cyclic-luteal activity were used. The cows were kept in pasture (*Brachiaria sp*) supplemented with corn silage, with ad libitum access to minerals and water.

2.2. Experimental design

At the first ultrasound examination all cows were in diestrus; therefore they were given sodium cloprostenol (500 μg im; Sincrocio; Ourofino Agronegocio, Sao Paulo, SP, Brazil) and received an ear implant containing norgestomet (3 mg; Crestar; Merck Animal Health, Boxmeer, The Netherlands). The emergence of a new follicular wave was synchronized by an estradiol benzoate treatment (2 mg im; Sincrodiol, Ourofino Agronegocio) immediately after implant insertion. Transrectal ultrasonography of the ovaries (Aquila Vet; Esaote, Genova, Italy) with an 8.0 MHz linear-array transducer was performed every 12 h and the follicles were individually monitored from emergence until aspiration, and further residual follicles were also monitored. The experimental design had two main factors: diameter of the follicle before aspiration and norgestomet treatment. Cows were randomly assembled to four treatment groups and aspiration was performed when the largest follicle reached the expected diameter of deviation (8 mm) or dominance (12 mm), in the presence or not (implant removed at wave emergence) of norgestomet treatment.

Follicle aspiration was performed with the same ultrasound device (Aquila Vet; Esaote), but with a microconvex 7.5 MHz transducer. The ultrasound image was used to guide a 20 ga disposable needle coupled to a Teflon circuit. The ovarian follicle aspiration procedure was performed as previously described [25]. A conventional OPU system was adapted to individually recover follicular fluid from the targeted follicles into 1.5 mL tubes. Follicular fluid was then centrifuged at 600 X *g* for 10 min to remove cells and cumulus-oocyte complexes, and the supernatant stored at −20 °C until radioimmunoassay analysis. After aspiration, collapsed follicular walls were monitored every 12 h and the presence of a fluid-filled antrum was referred to as a residual follicle (RF). The content of RF was collected 36 to 48 h after the first follicle aspiration, using the same procedures.

After follicle aspiration, each cow was assembled into a different group, in a crossover distribution. A total of 18 follicles were sampled in each group, for a total of 72 follicles (6.0 ± 0.9 per cow, ranging from 3 to 12).

2.3. Hormonal assays

Intrafollicular concentrations of estradiol and progesterone were determined by solid-phase I^{125} radioimmunoassay (RIA), using commercial RIA kits (TKE22 Coat-A-Count Estradiol and TKPG5 Coat-A-Count Progesterone, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) at the Endocrinology Laboratory of the College of Veterinary Medicine and Animal Science, São Paulo State University, São Paulo, SP, Brazil. If necessary, samples were diluted 1:10 to 1:1000 to fit the standard curves. Assay sensitivity was 0.02 ng/mL and 10 pg/mL for progesterone and estradiol, respectively. Inter- and intra-assay coefficients of variation were 2.75% and 1.87%, respectively, for progesterone; and 6.0% and 12.7% for estradiol. Quality control was performed according the manufacturer's instructions, using samples of known concentrations of

progesterone (0.1 and 2.7 ng/mL) and estradiol (26.3 and 510.0 ng/mL).

2.4. Residual follicle steroidogenic activity analysis

To classify RF according to steroidogenic activity, they were first ranked according estradiol concentrations, from the lowest to the highest value within a group. Mean and SD were calculated for each four consecutive values within a group. Thus, the first value was removed and the procedure was repeated for the consecutive four values, throughout the last estradiol value. Coefficients of variation (mean ± SD) were then estimated for each four consecutive values (N = 15) and compared with the estradiol intra-assay CV (12.7%). The edge between residual follicles with low or high concentrations of estradiol was determined when the first estimated CV was equal or higher than 2.5 times the intra-assay CV. The threshold value was considered the average of the four estradiol concentrations used to estimate this CV. Samples with concentrations above this value were considered to be from estradiol-active residual follicles. The same procedure was used to determine the progesterone activity and characterize luteinized residual follicles. Steroid-inactive follicles were characterized when both estradiol and progesterone concentrations were low.

2.5. Statistics

Data for follicle growth and intrafollicular concentrations of progesterone and estradiol were examined for normality using the Shapiro–Wilk test; no transformation was needed. The follicle development before aspiration at 8 or 12 mm was analyzed for the main effects of group and hour and their interactions. The SAS MIXED procedure with a repeated statement was used to account for the autocorrelation between sequential measurements. If a significant main effect of hour was detected, differences in mean follicle diameters were compared among hours using the least significant difference test. When a significant effect of group or interaction was detected, the differences among groups within an hour were assessed by Tukey's post hoc test. Single-point (follicle diameter before aspiration) or discrete data (intrafollicular concentrations of progesterone, estradiol, and estradiol [E2]:progesterone [P4] rate) were analyzed by the general linear models (GLM) procedure, with means compared among groups by Tukey's post hoc test, or between groups in the same follicle size by the Student *t* test. The percentages of RF in each group/category according to ovarian steroid concentrations were analyzed using the chi-square test. All statistical analyses were performed using the SAS software (The SAS System for Information Delivery, version 8.02, SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± SEM. Statistical significance was considered based on $P < 0.05$.

3. Results

Follicular growth was not affected by norgestomet treatment ($P > 0.05$; Fig. 1), thus the interval from emergence to aspiration of the largest follicle was similar between group 1 (G1) and group 2 (G2) (63.5 ± 4.5 vs. 65.3 ± 5.2 h, respectively; $P > 0.05$), and between group 3 (G3) and group 4 (G4) (95.0 ± 5.4 vs. 96.7 ± 5.3 h, $P > 0.05$).

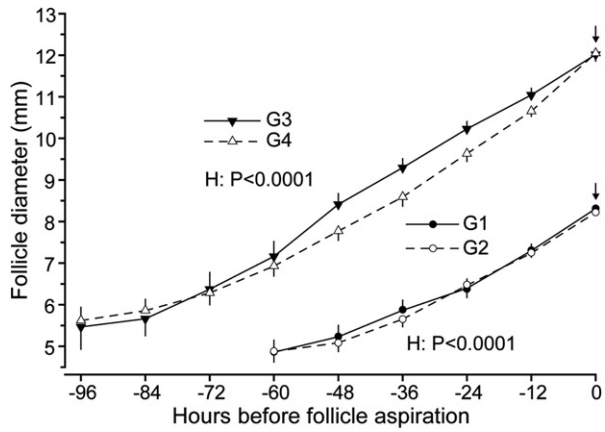

Fig. 1. Follicular development in cows treated (G1, G3) or not (G2, G4) with norgestomet implants, before aspiration of the largest follicle with 8 mm (G1, G2) or 12 mm in diameter (G3, G4). Black arrows indicate follicular aspiration.

One dominant follicle of the G4 group was later identified as atretic (E2:P4 ratio <1) and excluded from analysis, as well as its subsequent RF. Therefore, the number of follicles included on G4 was reduced to 17, and the total number of follicles in this study was 71. There were no differences ($P > 0.05$) in follicle diameter at aspiration nor in estradiol concentrations in follicular fluid between G1 and G2 or between G3 and G4 (Table 1). As expected, 12 mm follicles produced more estradiol than 8 mm follicles ($P < 0.05$). Norgestomet treatment, however, reduced intrafollicular progesterone concentration in 8 mm follicles (G1) and, consequently, increased E2:P4 ratio in this group (Table 1).

Based on ultrasonographic examinations of the follicular site after aspiration, 73.2% (52/71) of the remaining follicular walls were refilled with fluid (defined as RF). The antrum of the RFs was first visualized 12 to 24 h after follicle aspiration. There was no effect of norgestomet treatment on the occurrence of residual follicles (66.7% [12/18] vs. 61.1% [11/18] for G1 vs. G2, respectively; and 83.3% [15/18] vs. 82.4% [14/17] for G3 vs. G4, $P > 0.05$). A trend of increased occurrence of RF, however, was

Table 1
Follicle diameter and follicular fluid estradiol and progesterone concentrations among follicles aspirated before (G1 and G2, 8 mm) or after (G3 and G4, 12 mm) deviation, from cows treated (G1 and G3) or not (G2 and G4) with norgestomet.

Group	N	Follicle diameter (mm)	Estradiol (E2) ng/mL	Progesterone (P4) ng/mL	E2:P4
G1	18	8.3 ± 0.1^{a}	304.0 ± 64.2^{a}	5.8 ± 1.5^{b}	180.4 ± 77.9^{a}
G2	18	8.2 ± 0.1^{a}	266.1 ± 52.3^{a}	23.4 ± 7.1^{a}	22.8 ± 6.0^{b}
G3	18	12.0 ± 0.2^{c}	815.9 ± 150.6^{c}	37.8 ± 10.0^{c}	51.6 ± 11.5^{c}
G4	17	12.0 ± 0.1^{c}	831.2 ± 193.0^{c}	33.0 ± 5.8^{c}	38.8 ± 8.5^{c}

For groups G1 and G2, within a column, means without a common superscript letters (^{a,b}) differed ($P < 0.05$). For groups G3 and G4, within a column, means with a common superscript letter (^{c}) did not differ ($P > 0.05$).

Table 2
Diameter and steroidogenic patterns of residual follicles (RF) formed after follicular aspiration before (8 mm; G1 and G2) or after deviation (12 mm; G3 and G4), from cows treated (G1 and G3) or not (G2 and G4) with norgestomet.

Group	N	Diameter (mm)	Estradiol (E2) ng/mL	Progesterone (P4) ng/mL	E2:P4
G1	12	10.0 ± 0.5^{a}	555.0 ± 115.8^{a}	88.4 ± 42.2^{a}	23.7 ± 12.2^{a}
G2	11	9.1 ± 0.4^{a}	317.6 ± 150.1^{a}	26.6 ± 10.2^{a}	13.6 ± 4.9^{a}
G3	15	10.4 ± 0.4^{b}	900.9 ± 235.2^{b}	224.3 ± 114.0^{b}	28.1 ± 11.4^{b}
G4	14	11.1 ± 0.6^{b}	390.7 ± 217.8^{c}	467.0 ± 172.0^{b}	14.5 ± 8.9^{b}

For groups G1 and G2, within a column, means with a common superscript letter (^{a}) did not differ ($P > 0.05$). For groups G3 and G4, within a column, means without a common superscript letters (^{b,c}) differed ($P < 0.05$).

observed in 12 mm follicles, when compared with 8 mm follicles (82.8% [29/35] vs. 63.9% [23/36], respectively; $P = 0.07$), regardless of treatment. The interval from aspiration of the largest follicle to aspiration of the subsequent residual follicle was similar among groups (49.0 ± 5.0, 46.9 ± 6.4, 43.2 ± 4.8, and 39.4 ± 4.1 h for G1, G2, G3, and G4, respectively; $P > 0.05$).

The average diameter of the residual follicles was 10.2 ± 0.2 mm, and there was no significant difference between RF formed from groups of similar follicle diameters before aspiration (10.0 ± 0.5 vs. 9.1 ± 0.4 mm for G1 and G2; 10.4 ± 0.4 vs. 11.1 ± 0.6 mm for G3 and G4, respectively; $P > 0.05$). When compared with their original diameters, RF formed after aspiration of 8 mm follicles had an increase in size ($+1.7$ mm [$P < 0.01$] and $+0.9$ mm [$P < 0.05$] for G1 and G2, respectively), whereas those formed from 12 mm follicles had a decrease in G3 (-1.7 mm; $P < 0.01$) but not in G4 (-1.0 mm; $P > 0.05$).

The steroidogenic pattern of the fluid collected from residual follicles was first analyzed according to experimental groups. Estradiol concentration was lower in residual follicles formed after aspiration of 12 mm follicles in cows not treated with norgestomet (G4), compared with those from treated ones (G3; Table 2). It was noteworthy that G4 was the only group in which intrafollicular concentration of steroids was different in the residual follicles when compared with the former follicles (estradiol: -530.7 ± 133.9 ng/mL [$P < 0.01$]; progesterone, $+429.6 \pm 171.7$ ng/mL [$P < 0.05$]).

There were three possibilities for steroidogenesis in the RF, with remarkable differences among classes. The first one was residual follicles with high production of estradiol and high E2:P4 ratio, referred to as estradiol-active RF. The second included residual follicles with signs of luteinization, i.e., low estradiol but high progesterone, and consequently low E2:P4 ratio, referred to as luteinized RF. The third class consisted of residual follicles with low estradiol and progesterone, with an E2:P4 ratio close to 1:1, referred to as inactive RF (Table 3).

Retrospective analysis of steroidogenic activity in follicles that did not form RF, or developed into different RF categories (estradiol-active, inactive, or luteinized) highlighted some characteristics that might determine the fate of aspirated follicles (Table 4). Only 12 mm follicles underwent luteinization after OPU, all but one from G4 (5/6). Although 12 mm follicles were more likely to form RF, the proportion of follicles that later became estradiol-active RF was similar between follicles aspirated at 8 or 12 mm. Estradiol-active RFs, however, were formed from follicles with greater estradiol production, when compared with inactive RF, regardless of diameter.

4. Discussion

The occurrence of fluid-filled structures after ultrasound-guided follicle aspiration was previously reported by us and other groups [26,27,30]. In most cases, however, this was a secondary finding, because the primary goals of these studies were to recover cumulus-oocyte complexes, or simply to remove large antral follicles from the ovaries, promoting the emergence of a new follicular wave. Therefore, this phenomenon and its possible consequences were poorly described, and there is still no consensus regarding appropriate terminology. Therefore, the present study was designed to bring new insight regarding factors related to the frequency of occurrence and steroidogenic activity of fluid-filled structures formed after ultrasound-guided follicle aspiration, herein referred to as RF.

Similar to previously reports [27], in the current study the occurrence of residual follicles was not occasional, with frequencies exceeding 60% in all groups. This phenomenon seemed to be closely related to follicular size rather than LH support, because the percentage of RF arising after follicle aspiration was not affected by norgestomet treatment, although there was a trend to form a greater number of RF in 12 mm follicles, compared with 8 mm follicles. Whether or not RF occur might be related to the physical damage caused by the puncture on the follicular wall, an effect that would theoretically be greater in small follicles, in which the antrum is proportionally smaller [31] and the tip of the needle would easily reach the cells of the follicular wall. Interestingly, in the present study, although RFs formed from 8 mm follicles were less frequent, they were larger than the original follicles, demonstrating that the physical damage did not compromise developmental capacity. The association between physical damage and the fate of the

Table 3
Diameter and steroidogenic features of the residual follicles (RF) when categorized according to their steroidogenic pattern into estradiol-active, luteinized, or inactive.

Class of RF	Category (N) 8/12 mm	Diameter (mm)	Estradiol (E2) ng/mL	Progesterone (P4) ng/mL	E2:P4
Estradiol-active	17/17	10.8 ± 0.3^{a}	833.6 ± 128.7^{a}	130.2 ± 52.4^{b}	30.3 ± 7.0^{a}
Luteinized	0/6	10.6 ± 0.8^{a}	1.6 ± 0.8^{b}	1078.4 ± 232.6^{a}	0.0 ± 0.0^{b}
Inactive	6/6	8.4 ± 0.4^{b}	33.0 ± 12.8^{b}	30.0 ± 8.1^{b}	1.1 ± 0.3^{b}

Within a column, means without a common superscript letters differed ($P < 0.05$).

Table 4
Follicular features before aspiration and different outcomes related to residual follicle (RF) formation.

Fate after aspiration	Category (N) 8/12 mm	Diameter (mm)	Estradiol (E2) ng/mL	Progesterone (P4) ng/mL	E2:P4
No RF formed	13/6	9.4 ± 0.4^{a}	$364.6 \pm 87.2^{a,b}$	17.7 ± 7.3	$46.2 \pm 9.6^{a,b}$
Estradiol-active RF	17/17	$10.2 \pm 0.3^{a,b}$	770.2 ± 118.8^{b}	27.5 ± 6.0	68.7 ± 14.3^{b}
Luteinized RF	0/6	11.7 ± 0.3^{b}	$337.5 \pm 118.1^{a,b}$	30.5 ± 14.4	$43.1 \pm 20.0^{a,b}$
Inactive RF	6/6	$10.2 \pm 0.6^{a,b}$	328.4 ± 132.6^{a}	29.0 ± 5.4	24.3 ± 14.4^{a}

Within a column, means without common superscript letters differed ($P < 0.05$).

aspirated follicles, however, may also be affected by a number of other factors, such as needle diameter, vacuum pressure, and aspiration procedure. In the present study, even though we used a standard procedure for cumulus oocyte complex recovery, with sharp and thin disposable needles (20 ga), and the follicular damage was expected to be less than from studies that used thicker needles (17 ga) and follicle wall scraping [26], RF were frequently formed.

In the current study, production of steroids by the remaining follicular wall cells was not interrupted by aspiration of follicle contents. This possibility was first hypothesized by Pieterse et al. [32], and later confirmed by our group [27]. In our previous study, however, RFs were observed in cows undergoing repeated OPU, and were formed from follicles aspirated in different developmental stages. The current study was thus designed to evaluate steroidogenic activity in RF formed under controlled conditions, in follicles aspirated during their growth phase, before or after deviation, and with or without norgestomet exposure (to indirectly regulate LH stimulation).

Despite the lack of norgestomet effect on the occurrence of RF, a possible increased LH support may have affected follicular wall steroidogenesis after aspiration of post-deviation follicles (12 mm), because estradiol was reduced and progesterone increased in RF formed in the group not treated with norgestomet (G4). Follicle deviation is associated with a shift in follicular dependence on gonadotrophic stimulation, from FSH to LH [7], and because norgestomet treatment reduces LH secretion [33], it was expected to affect mainly the 12 mm follicles (G3). The absence of this negative feedback might have increased LH pulses and induced luteinization, which occurred mainly in the G4 group. In that regard, follicles aspirated after a preovulatory LH surge are more likely to form luteal tissue [29].

Individual analysis of estradiol and progesterone contents in the fluid collected from each RF fluid indicated three possible fates for these structures: (1) maintain high estradiol production and, consequently, a high E2:P4 ratio, similar to regular growing follicles; (2) undergo luteinization, with a clear shift to progesterone secretion; and (3) reduce steroidogenic activity. We, therefore, proposed their classification as estradiol-active, luteinized, or inactive RF. In the latter, we hypothesize that mural cells underwent atresia after aspiration, and the recovered fluid was composed mainly of serum from the retraction of blood clots within the aspirated follicles, instead of driven to the antrum by granulosa cell secretion, as in healthy follicles [31]. The category estradiol-active RF was the most frequent outcome (65.4%), both in follicles close to the expected size of deviation (8 mm) and in dominant follicles postdeviation (12 mm), demonstrating that follicle aspiration does not immediately disrupt estradiol production by the remaining follicular wall cells. Consequently, estradiol-active RF may potentially affect, at least for the period evaluated in the present study (48 h), follicular dynamics after follicular aspiration.

Although an E2:P4 ratio >1 is generally considered an indicator of follicular viability [34], a high variation in steroid concentration has been reported in the follicular fluid of regular growing follicles [34–36]. These variable results might be partially related to the specificity and the sensitivity of the hormonal assays, but they also reflect differences in follicular developmental potential. In the present study, all aspirated follicles were classified as viable (E2:P4 ratio >1), but the concentrations of steroids in RF were highly variable among groups. To better understand how the follicle status could be linked to the occurrence of RF, we retrospectively analyzed steroidogenesis in the original follicles that later became one of the RF classes (estradiol-active, luteinized, or inactive). Consistent with the previous comparison among groups, there was an association between follicle diameter and its fate after aspiration. The most interesting finding, however, was related to intrafollicular concentrations of estradiol. Although all follicles were classified as viable, those with highest estradiol concentrations were more likely to form estradiol-active RF. Estradiol production is related to follicular development potential, because the further dominant follicle usually presents higher intrafollicular concentrations of estradiol before deviation than the largest subordinate follicle [35]. Thus, differences in developmental potential of the follicles before aspiration probably affect their fate thereafter.

Follicle ablation by ultrasound-guided follicle aspiration was proposed as an alternative to replace procedures such as eletrocauterization or irradiation to remove the largest follicles and to eliminate the effects of follicular dominance [13,32]. In fact, it is much less invasive, expensive, and time-consuming than other approaches, and can be performed in experimental and in field conditions. In most of the recent studies with follicle ablation, ultrasound-guided follicle aspiration was used to eliminate the dominant follicle [9,12–19]. Eletrocauterization, however, results in immediate destruction of the granulosa cells and, consequently, interruption of estradiol production [32], whereas follicle content aspiration does not, as demonstrated in the present study.

Based on the present experiment, we can now confirm that even though atresia is the fate of all nonovulated follicles [37], this will not necessarily occur immediately as a consequence of ultrasound-guided follicular aspiration. Moreover, there is evidence that the remaining follicular wall from punctured follicles can either remain estradiol-active or

subsequently undergo luteinization. Before complete atresia takes place, residual follicles may produce variable amounts of estradiol and/or progesterone, and potentially affect the endocrine balance and further follicular dynamics. These findings may explain, for example, the higher incidence of follicular cysts (Viana, unpublished data), subluteal progesterone concentrations [28], and abnormal follicular growth patterns [25,38] in cows undergoing repeated OPU. Furthermore, inconsistencies observed in previous studies that used follicle ablation by ultrasound-guided aspiration, such as lack of significant reduction in plasma estradiol after follicular puncture [28], and failure to improve the production of viable embryos after superovulation [18,39,40], could be related to occurrence of RF. Only a few studies considered in their methodology the possibility of the RF formation after follicle ablation and an eventual interference in follicular dynamics. Ginther et al. [26], for example, excluded data when follicle refilling was observed in heifers, and Gastal et al. [30] reablated follicles that had the antrum refilled after first ablation in mares. A possible general effect of RF in previous studies using follicle aspiration may be difficult to infer, because there was no standard in ablation procedures (needle type, vacuum pressure, number of follicles removed, etc.), but it shall not be neglected in the interpretation of subsequent findings in future experiments of this kind.

In conclusion, fluid-filled structures with variable patterns of steroid production, referred to as residual follicles, were frequently formed after ultrasound-guided follicular aspiration. The occurrence and functional characteristics of residual follicles depend on the diameter and status of the former punctured follicle, and also on systemic gonadotrophic support.

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